Maternal rs1801131 genotype in *MTHFR* modifies the association between prenatal folate levels and child's developmental delay.

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Äidin rs1801131 genotyyppi *MTHFR* geenissä moderoi äidin raskauden aikaisten folaattitasojen yheyttä lapsen kehityksen viivästymään.

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Tiivistelmä – Abstrakt – Abstract

Tausta: Folaatti on välttämätön ravintoaine neurokehitykselle. Folaattiaineenvaihduntaan vaikuttaa geneettinen variaatio methylenetetrahydrofolate reductase (*MTHFR*) geenissä.Tutkielman tarkoituksena on selvittää vaikuttaako äidin *MTHFR* geenin kolme yhden emäksen monimuotoisuutta (SNP) lapsen kehitysviiveen riskiin ja muokkaako genotyyppi folaattitason vaikutusta lapsen kehitysviivästymän riskiin.

Metodit: Tutkielmassa käytettiin osa-aineistoa Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction (PREDO) -kohortista. Folaattitasot mitattiin äidin veren seerumista korkeintaan kolme kertaa raskauden aikana. Äidin *MTHFR* SNPs (rs1801133, rs1801131, rs1999594) genotyypitys tehtiin käyttäen Global Screening Array sirua. Lapsen kehitysviivettä mitattiin käyttäen Ages and Stages Questionnaire (ASQ-3) –kyselyn kolmatta painosta, jonka äidit täyttivät lapsen ollessa 2 – 5 -vuotiaita. Kehityksen viiveen suuruus mitattiin monessako kehityksen osa-alueessa lapsi ei saavuttanut odotettua kehitystä.

Tulokset: Äidin raskausajan folaattitasot eivät olleet yhteydessä lapsen kehitysviivästymään. Äidin rs1801131 AC/CC genotyyppi oli yhteydessä korkeampaan äidin folaattitasoon ja vähäisempään kehitysviivästymään lapsella. Äidin rs1801131 genotyyppi muokkasi äidin alkuraskauden aikaisten folaattitasojen vaikutusta lapsen kehitysviivästymään. Äidin AC/CC genotyyppi vähensi kehitysviiveen riskiä matalilla folaattitasoilla verrattuna AA ryhmään. Riippuen mallista, korkeilla folaattitasoilla joko ei havaittu merkitsevää eroa, tai AC/CC ryhmään kuuluvien äitien lapsilla havaittiin hieman korkeampi riski verrattuna AA ryhmään riippuen mallista. Äidin rs1801133 CC genotyyppi oli yhteydessä korkeampaan folaattitasoon kuin CT/TT kaikissa muissa mittausajankohdissa paitsi alkuraskaudesta. Äidin rs1999594 ei ollut yhteydessä folaattitasoihin. Yhteyksiä ei löydetty myöskään äidin rs1801133 tai rs1999594 genotyyppien ja lapsen kehitysviiveen välillä.

Johtopäätökset: Äidillä oleva rs1801131 C alleeli voi suojata lasta kehitysviiveeltä erityisesti silloin, kun äidin alkuraskauden aikainen folaattitaso on matala. Toisaalta se saattaa olla myös riskitekijä korkeilla folaattitasoilla.

Avainsanat – Nyckelord – Keywords
Kehityksen viive, raskaudenaikiainen folaatti, *MTHFR*

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Tiivistelmä / Abstrakt – Abstract

Background: Micronutrient folate is crucial in neural development. Folate metabolism is affected by genomic variation in the methylenetetrahydrofolate reductase (*MTHRF*) gene. This study set out to study the effects of prenatal maternal folate levels and genomic variation in *MTHFR* on offspring developmental delay (DD), and whether the candidate maternal single nucleotide polymorphisms (SNPs) modify the effect of folate on the risk of DD in the offspring.

Methods: The mother-child dyads in this study are part of the Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction (PREDO) -cohort. Folate levels were measured from the serum up to three times during pregnancy. The maternal *MTHFR* SNPs (rs1801133, rs1801131, rs1999594) were genotyped using the Illumina Global Screening Array. The children's developmental milestones were assessed using the Ages and Stages Questionnaire Third edition (ASQ-3) completed by the mothers between the years 2 and 5. Developmental delay was measured as the number of categories where the child did not reach expected development.

Results: Maternal folate levels during pregnancy did not associate with the risk of DD in the offspring. Any C alleles in the maternal rs1801131 associated with both higher folate levels during pregnancy in the mother and a lower risk of DD in the offspring. Moreover, maternal rs1801131 modified the association between the early pregnancy folate levels and the risk of DD in the offspring (p-value for interaction < .05). Increasing maternal early pregnancy folate levels increased the risk of DD in the offspring with maternal C allele carriers and decreased with maternal AA carriers. While the CC genotype in rs1801133 associated with higher folate levels during pregnancy in the mother, we found no associations between maternal rs1801133 or rs1999594 and the risk of DD in the offspring.

Conclusions: The current findings suggest that C alleles in the *MTHFR* SNP rs1801131 may protect early neurodevelopment in the offspring, especially when folate levels are low, but might also be a risk factor with high folate levels.

Avainsanat / Nyckelord – Keywords

Developmental delay, prenatal folate, *MTHFR*

Säilytyspaikka / Förvaringsställe – Where deposited

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Contents

1. Introduction

Developmental delay (DD) is considered when a child does not meet one or more developmental milestones at an appropriate age after taking into account the broad variation of typical development (Rydz, D., Shevell, Majnemer & Oskoui, 2005). Approximately 15% of children in the U.S. display any DD with boys having almost a twofold risk compared to girls (Boyle et al., 2011). DD has been connected to adverse outcomes, for example children with DD have an increased risk of developing behavioural and emotional problems later in life (Crnic, Hoffman, Gaze, & Edelbrock, 2004; Herring, Gray, Taffe, Tonge, Sweeney, & Einfeld, 2006). Maternal nutrition during pregnancy has been found to affect child's neurocognitive development (Monk, Georgieff & Osterholm, 2013) and one important micronutrient for neural development during pregnancy is folate (Czeizel, & Dudás, 1992). It has also been suggested to play a role in neurocognitive development (Gao et al., 2016).

Folate, also known as vitamin B9, is needed in various cellular processes such as DNA methylation (Bagley & Selhub, 1998; Bodnar et al., 2010; Greenberg et al., 2011). It also plays a key role in fetal development as it is needed in large amounts for cell growth and divisions (Taparia, Gelineau-van Waes, Rosenquist, & Finnell, 2007). Folate deficiency during pregnancy can lead to serious malformations in neurodevelopment as indicated by the well-established association between folate deficiency during pregnancy and neural tube defects (NTDs) in the fetus (Czeizel, & Dudás, 1992; Milunsky et al.., 1989; Wolff, Witkop, Miller & Syed, 2009). Due to its importance during pregnancy, it is recommended to increase the intake of folate before and during pregnancy (Terveyden ja Hyvinvoinnin Laitos [THL], 2018; World Health Organization [WHO], 2019). Naturally occurring folate isoforms can be obtained from food, including leafy vegetables, while folate supplements typically contain the synthetically produced isoform often referred as folic acid.

Previous research has linked maternal serum folate levels during pregnancy to differences in neurocognitive development in the offspring (Schlotz et al., 2010; Steenwegde et al., 2012; Veena et al., 2010), yet not constantly (Campoy et al., 2011; Steenweg-de Graaff et al., 2014; Tamura, 2005; Wu, Dyer, King, Richardson & Innis, 2012). However, previous research has not been consistent with the timing of folate level measurement as it varies between studies from the early second trimester to the end of third trimester (Campoy et al., 2011; Schlotz et al., 2010; Steenwegde et al., 2012; Steenweg-de GraaffJ et al., 2014; Tamura, 2005; Veena et al., 2010; Wu et al., 2012). Further, only two studies (Campoy et al., 2011, Tamura, 2005)

have used multiple measurement times. One explanation for the varying results could be the possible modifying effect of folate related genes.

Methylenetetrahydrofolate reductase (MTHFR) enzyme, encoded by the *MTHFR* gene, is a key part of the folate cycle as it converts 5, 10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). 5-methylTHF is the primary circulating form of folate and is a methyl donor for the methyl cycle (Blom & Smulders, 2011). Single nucleotide polymorphisms (SNPs) in the *MTHFR* affect the MTHFR enzyme and trough that they affect the amount of circulating folate.

The most studied SNP in the *MTHFR* is rs1801133. TT carriers in rs1801133 have 35% residual activity of the MTHFR enzyme compared to CC carriers (Frosst et al., 1995) and the T allele in rs1801133 is further linked with lower folate concentrations (Deng et al., 2018; Grarup et al., 2013; van der Put et al., 1998). Maternal T alleles have also been connected to poorer mental development in the offspring (Pilsner et al., 2010). The observed association presented above is thought to be mediated by the impaired enzyme function caused by the T allele which affects the folate levels through impaired folate metabolism leading to disturbed development (Pilsner et al., 2010). The findings reported by Del Rio Garcia et al. (2009) support the hypothesis as they found that low prenatal folate intake is especially harmful for child's neurodevelopment with mothers having the TT genotype. However, some recent findings suggest a more complex interaction between the maternal rs1801133 and prenatal folate levels. Gatica-Domínguez et al. (2019) found that increased maternal prenatal folate level was inversely associated with child's mental development only in the group of mothers carrying CC genotype. Combining these findings, it is possible that maternal prenatal folate levels place a U-shaped risk on developmental problems and the *MTHFR* genotype affects whether a person is in greater risk for adverse effects associated with too low or too high folate levels during pregnancy (Gatica-Domínguez et al., 2019).

The second most studied SNP in *MTHFR* is rs1801131 which also has been observed to lower MTHFR enzyme activity yet in a lesser manner than rs1801133 (van der Put et al., 1998; Weisberg et al., 2001; Weisberg, Tran, Christensen, Sibani & Rozen, 1998). However the association between rs1801131 and folate metabolism may be more complex as a recent meta-analysis (Yu et al., 2018) concluded that the C allele in rs1801131 is connected to higher folate concentrations in the general population, excluding elderly population in which the C allele did not seem to have any effect. The C allele in children has been connected to a

decreased risk for autism spectrum disorders (ASD) (Pu et al., 2013) thus indicating some importance of the rs1801131 genotype in the child's development even though maternal rs1801131 has not been previously associated to neurocognitive development in the offspring (del Río Garcia et al., 2009; Pilsner et al., 2010).

In addition rs1999594, which is located in the regulatory region of the *MTHFR*, has shown some association with folate levels in one relatively small genome-wide association (GWA) study (Tanaka et al, 2009), but a larger one did not replicate the association (Hazara, 2009). However, rs1999594 showed an association with increased homocysteine level, which is an indirect indicator of low folate levels, in two large GWA studies (Hazara, 2009; Paré et al., 2009), but not in a relatively small GWA study (Lange et al., 2010). To my knowledge, no prior study has asses the effect of maternal rs1999594 on child's development.

The aim of this study is to examine whether (i) prenatal folate levels associate with the risk of DD in the child and whether (ii) maternal *MTHFR* SNPs (rs1801133, rs1801131, rs1999594) associate with folate levels and (iii) the risk of DD in the child and (iv) whether these three SNPs modify the association of maternal folate level during pregnancy on child's DD.

1.2 Research questions and hypotheses

1. Do prenatal folate levels associate with the risk of DD in the child?

Hypothesis: Low serum folate levels throughout pregnancy are associated with an increased risk of DD.

2. Are rs1801131, rs1801133 and rs1999594 associated with serum folate levels?

Hypotheses: The T allele in rs1801133 is associated with decreased folate levels, rs1801131 C allele with increased folate levels. For rs1999594 no hypotheses are possible to place due to lack of pre-existing literature.

3. Are maternal polymorphisms in rs1801131, rs1801133 and rs1999594 associated with child's risk of DD?

Hypotheses: The T allele in rs1801133 is associated with an increased risk for DD. For rs1801131 the results from prior studies are too contradictory to place well-founded hypotheses and for rs1999594 no prior literature exists and therefore no hypothesis is possible to place.

4. Do maternal *MTHFR* SNPs rs1801131, rs1801131 and rs1999594 moderate the association between prenatal folate levels during different pregnancy trimesters and offspring DD during childhood?

Hypotheses: The maternal rs1801133 T allele together with low maternal prenatal folate levels and the C allele with high maternal prenatal folate levels increase the risk for DD in the offspring. There is too little research to place hypotheses for the other two loci.

2. Methods

2.1 Participants

The samples used in this study are part of the genetic sub-study of Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction (PREDO) study. Pregnant women were recruited from 10 study clinics between 2005 and 2009 when they had their first ultrasound screening at 12^{+6} – 13^{+6} weeks of pregnancy. The final sample for the original PREDO study consisted of 4777 women who gave written consent and gave birth to singleton live born children. Up to date three participants have withdrawn their consent and therefore the original PREDO sample consist of 4774 participants at the moment. Further information of the study sample is available in Girchenko et al. (2017).

Of the original sample 1020 mothers (21%) had genotype information and written consent. From those participants 456 mother-child pairs participated in the 3.5 years follow-up and filled the Ages and Stages questionnaire (ASQ) within the appropriate developmental time period and returned written consent, thus forming the study sample 1 (Table 1).

Blood samples during pregnancy for the folate assays were collected from 443 mothers, 440 of which had the serum folate concentration available on at least one timepoint during pregnancy. Of those mothers, 415 had also genotype data available. This sample formed the study sample 2 (Table 2). Of those 415 participants, 184 children had ASQ information within the appropriate age period and those mother-child pairs formed the study sample 3 (Table 1). 192 participants had information of prenatal folate levels from at least one timepoint, child's ASQ questionnaire filled at the appropriate age but no genotype information. Those mother-child pairs formed the study sample 4. We did not conduct separate characteristics of the sample or attrition analysis for the sample 4 as there were only 8 mother-child pairs in the sample 4 who were not included in study sample 3.

Table 1. *Characteristics of the study samples and attrition analysis.*

	Sample 1 (genotype and ASQ data)		Sample 2 (folate and genotype data)		Sample 3 (folate, ASQ, and genotype data)		Original PREDO sample
	M (SD)/ n (%)	p[a]	M (SD)/ n (%)	p[a]	M (SD)/ n (%)	p[a]	Mean (SD)/ n (%)
Size of the sample	456 (9.6%)		415 (8.7%)		184 (3.9%)		4774 (100%)
Mother's Characteristics							
Maternal age at delivery (years)	33.5 (5.7)	< **.001**	32.5 (5.3)	< **.001**	32.4 (5.6)	**.02**	31.5 (4.9)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		10 (0.2%)
Mother's highest education level		.87		**.004**		.32	
Secondary	190 (41.7%)		198 (47.7%)		84 (45.7%)		1802 (37.7%)
Lower tertiary	113 (24.8%)		89 (21.4%)		40 (21.7%)		1137 (23.8%)
Upper tertiary	153 (33.6%)		122 (29.4%)		60 (32.6%)		1452 (30.4%)
Data not available n (%)	0 (0%)		6 (1.4%)		0 (0%)		383 (8.0%)
Smoking during pregnancy		< **.001**		**.004**		.10	
No	456 (95.6%)		387 (93.3)		174 (94.6%)		4341 (90.9%)
Quit during first trimester	15 (3.3%)		19 (4.6%)		7 (3.8%)		167 (3.4%)
Throughout pregnancy	5 (1.1%)		8 (1.9%)		3 (1.6%)		240 (5.0%)
Data not available n (%)	0 (0%)		1 (0.2%)		0 (0%)		26 (0.5%)
Mother's metabolic disorders							
Score if any metabolic disorder		.21		**.02**		**.01**	
No	144 (31.6%)		138 (33.3 %)		68 (37.0 %)		318 (77.4%)
Any	312 (68.4%)		276 (66.5)		116 (63.0 %)		761 (15.9%)
Data not available n (%)	0 (0%)		1 (0.2%)		0 (0%)		3695 (77.4%)
Hypertensive disorders		< **.001**		< **.001**		< **.001**	
Normotension	244 (53.5%)		211 (50.8%)		100 (54.3 %)		3758 (78.7%)
Gestational hypertension	40 (8.8%)		38 (9.2%)		17 (9.2%)		272 (5.7%)
Pre-eclampsia	43 (9.4%)		42 (10.1%)		15 (8.2%)		209 (4.4%)
Pre-Pregnancy / Chronic hypertension	67 (14.7%)		67 (16.1%)		26 (14.1%)		200 (4.2%)
Data not available n (%)	62 (13.6%)		57 (13.7%)		26 (14.1%)		319 (6.7%)
BMI		< **.001**		< **.001**		< **.001**	
Underweight	12 (2.5%)		12 (2.9%)		7 (3.8%)		159 (3.3%
Normal weight	220 (48.2%)		193 (46.5%)		94 (51.1%)		2998 (62.6%)
Overweight	74 (16.2%)		87 (21.0%)		38 (20.7%)		930 (19.5%)
Obese	150 (32.9%)		123 (29.6%)		45 (24.5)		672 (14.1%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		25 (0.5%)
Maternal diabetes		< **.001**		< **.001**		< **.001**	
No diabetes	319 (70.0%)		274 (66.0%)		132 (71.7%)		4033 (84.5%)
GDM	113 (24.8%)		105 (25.3%)		42 (22.8%)		545 (11.4%)
Type 1	8 (1.8%)		10 (2.4%)		4 (2.2%)		28 (0.6%)
Type 2 or other by child birth	16 (3.5%)		26 (6.3%)		6 (3.3%)		143 (3.0%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		25 (0.5%)
Mother's genotype							
rs1801131		.68		.78		.76	
AA	214 (46.9%)		196 (47.2%)		89 (48.4 %)		468 (9.8%)
AC	199 (43.6%)		182 (43.9%)		79 (42.9 %)		458 (9.6%)
CC	43 (9.4%)		37 (8.9%)		16 (8.7 %)		94 (2.0%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		3754 (78.6%)

rs1801133		.51		.76		.87	
CC	262 (57.5%)		236 (56.9%)		101 (54.9%)		576 (12.1%)
CT	171 (37.5%)		151 (36.4%)		71 (38.6%)		383 (8.0%)
TT	23 (5%)		28 (6.7%)		12 (6.5%)		61 (1.3%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		3754 (78.6%)
rs1999594		.14		.96		.98	
AA	183 (57.5%)		157 (27.8%)		68 (37.0%)		381 (8.0%)
AG	214 (46.9%)		200 (48.2%)		90 (48.9%)		492 (10.3%)
GG	59 (12.9%)		28 (6.7%)		26 (14.1%)		147 (3.1%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		3754 (78.6%)
Metabolic data during pregnancy							
First measurement folate	17.2 (1.9)	.69	17.0 (7.6)	.49	17.2 (1.9)	.65	17.0 (7.6)
Data not available n (%)	278 (61.0%)		16 (3.9%)		6 (3.3%)		4352 (91.2)
Second measurement folate	15.2 (7.8)	.43	14.8 (7.7)	.38	15.2 (7.8)	.42	14.8 (7.6)
Data not available n (%)	274 (60.1%)		7 (1.7%)		2 (1.1%)		4342 (91.0%)
Third measurement folate	13.4 (7.6)	.49	13.0 (7.0)	.13	13.4 (7.6)	.44	13.1 (7.0)
Data not available n (%)	277 (60.7%)		16 (3.9%)		5 (2.7%)		4350 (91.1%)
Mean folate level	15.2 (7.3)	.56	14.9 (6.9)	.32	15.2 (7.3)	.51	15.0 (6.9)
Data not available n (%)	272 (59.6%)		0 (0%)		0 (0%)		4334 (91.0%)
Child's characteristics							
Gestational age	39.7 (1.8)	**.01**	39.5 (2.0)	< **.001**	39.7 (2.0)	.08	39.9 (1.6)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		4 (0.08%)
Sex		.59		.52		.44	
Boy	233 (51.1%)		224 (54.0%)		91 (49.5%)		2484 (52.0%)
Girl	223 (48.9%)		191 (46.0%)		93 (50.5%)		2273 (47.6%)
Data not available n (%)	0 (0%)		0 (0%)		0 (0%)		17 (0.4%)
Child's age at follow-up (months)	44.0 (8.6)	< **.001**	44.7 (10.9)	**.001**	44.7 (10.9)	**.001**	42.1 (8.2)
Data not available n (%)	0 (0%)		231 (55.7%)		0 (0%)		2356 (49.4%)
Mild developmental delay	0.6 (1.0)	.99	0.6 (1.1)	.78	0.6 (1.1)	.78	0.6 (1.0)
Data not available n (%)	0 (0%)		231 (55.7%)		0 (0%)		2356 (49.4%)
Failing to meet typical development	0.2 (0.7)	.93	0.2 (0.6)	.64	0.2 (0.6)	.64	0.2 (0.7)
Data not available n (%)	0 (0%)		231 (55.7%)		0 (0%)		2356 (49.4%)

Abbreviations: BMI, body mass index; GDM, gestational diabetes mellitus; p, p-value; M, mean; n, sample size; SD, standard deviation

[a] Results for attrition analysis comparing participants and non-participants as p-values from student t-test for continuous variables and Chi square test for categorical variables.

The results of the attrition analysis are described in Table 1. Compared to those not in the study samples, the mothers in all study samples were older, more likely to be obese, to have some hypertensive disorder and to have some type of diabetes. Also, compared to those not in the study samples, children in the study samples were older during the follow-up. In the samples 1 and 2 the mothers smoked less, and the pregnancies were shorter compared to those not in the study samples. In the study samples 2 and 3 the mothers had more often some metabolic disorder compared to those not in the study samples. And finally, in sample 2

mothers had lower highest education level compared to those mothers not in the study sample.

The study protocol for the original PREDO study was approved by the Ethics Committee of Obstetrics and Gynaecology and Women, Children and Psychiatry of the Helsinki and Uusimaa Hospital District and by the participating hospitals.

2.2 Measures

SNP genotyping

In total, 1066 mothers were genotyped with the Illumina Global Screening Array containing ~960.000 SNPs. Before imputation with Impute2 against the 1000Genomes reference panel, those with cell rate below 95%, discrepancy between phenotypic and genotypic sex, heterozygosity, or relatedness as well as population outliers were removed. After this QC, 1054 mothers were available in the final dataset.

Rs1801131, rs1801133, and rs1999594 were extracted from this data. The Hardy–Weinberg equilibrium (HWE) was tested and the observed frequency did not deviate from HWE ($p > .01$). The minor allele frequencies (MAFs) for rs1801131, rs1801133 and rs1999594 were 0.32, 0.24 and 0.38, respectively. The two loci in *MTHFR*, rs1801131 and rs1801133, are in linkage disequilibrium (LDE) with $D' = 1$ and $R^2 = 0.175$ in the Finnish sample (Machiela & Chanock, 2015). Rs1999594 is in linkage equilibrium with both rs1801131 and rs1801133.

For the analyses we decided to combine the heterozygous and homozygous groups for the minor allele as the group homozygous for the minor allele was small (see Table 1).

Developmental delay

Child's developmental outcomes were assessed by the mother using a Finnish translation of the third edition of the Ages and Stages questionnaire (ASQ-3) (Squires & Bricker, 2009) approved by the publisher. The questionnaire has been found to be reliable and valid with great sensitivity and specificity for screening children who are in need for further developmental follow-up and assessment (Squires, Bricker & Potter, 1997). The ASQ-3 questionnaire has 30 age groups and all groups have 30 age appropriate items to evaluate the development during different developmental period and the current study used the ones appropriate for children 2 to 5 years of age. The final scale of the ASQ-3 consists of five

developmental domains including fine motor, gross motor, problem solving, communication and personal-social skills. Each domain has six questions of whether the child can master the skill with responses “not yet”, “sometimes” and “yes” scored in order as 0, 5, 10. Sum of the scores in each domain vary between 0 and 60 depending whether the child can master none of the age specific skills (score 0) or all of the skills (score 60).

As there are no Finnish norms available for the ASQ-3, the delay in each domain was assessed by using the mean of the age-specific groups in this sample. Children scoring ≤ -1SD from the age specific mean were consider having at least mild DD in that specific domain as scoring between -1SD and -2SD has previously been used to reflect mild DD (Girchenko et al., 2018). Children whose score was ≤ -2SD from age specific mean were considered failing to meet typical development (FTD) in that domain. Children whose score was > -1SD were considered to be typically developed. Sum of the domains with at least mild DD reflected the wideness of at least mild DD across the domains. The score varied from 0 if the child did not have even mild DD in any of the five domains to 5 if the child had at least mild DD in each domain. The sum of the domains in which the child displayed FTD reflected the wideness of severe developmental delay across the domains. The score varied from 0 if the child met all the developmental milestones to 5 if the child did not meet typical development in any of the domains.

Serum folate level

Folate levels were measured from blood serum, which was collected three times during pregnancy at an early, mid and late timepoint. The serum folate was analyzed with the Architect 2000i immunoanalyzer (Abbott Diagnostics, Illinois, USA) at the Helsinki University Central Hospital (HUCH) laboratory according to standard practices. The serum folate level was measured as nmol/l. All participating mothers did not have data from all three measurement points and for that reason the mean folate level was calculated as the mean of all available timepoints. Table 2 presents the descriptive statistics of the different timepoints in which folate was measured. On average, the early measurement was done at the end of the first trimester, the mid measurement point was in the middle of the second trimester and the late measurement point was in the beginning of the third trimester. The folate levels in different timepoints were standardized.

Table 2. *Descriptive statistics of the folate measurement during pregnancy.*

	Folate level						Time of the measurement				
	Mean (nmol/l)	SD	Min	Max	Mothers with folate deficiency[a]	N	Mean (weeks)	SD	Min	Max	N
Early pregnancy	17.0	7.6	3.9	37.7	9.20 %	422	13.0	0.6	11.1	15.9	399
Mid pregnancy	14.8	7.6	2.4	44.2	21.60 %	432	19.3	0.6	17.1	22.9	410
Late pregnancy	13.1	7.0	3.3	37.4	31.00 %	424	27.1	0.8	19.6	31.1	403

Abbreviations: min, minimum; max, maximum, SD, standard deviation
[a]percentage of mothers with folate deficiency according to the measurement laboratory HUSLAB, retrieved from: https://huslab.fi/ohjekirja/1416.html, accessed on 25.2.2020

Covariates

The child's age when the ASQ-3 was filled was measured by months, weeks and days from birth. As children develop quickly at the age in which the ASQ-3 was filled was taken into account to control for the difference in development between first- and last-born children within each ASQ-3 age group. Male sex (e.g. Hintz et al., 2006; de Moura et al., 2010).), preterm birth (for review: Allotey et al., 2018), low maternal education (e.g. Demirci & Kartal, 2016; de Moura et al., 2010), maternal smoking during pregnancy (e.g. Clifford, Lang & Chen, 2012; Huizink & Mulder, 2006; Polanska Jurewicz, & Hanke, 2015) and maternal metabolic disorders (Girchenko et al., 2018) have been associated with increased risk for DD and hence were chosen as covariates.

Data on child's sex, (1: male, 2: female), gestational age, maternal education (secondary level or below, lower tertiary or at least upper tertiary), maternal smoking during pregnancy (non-smokers, those who quitted during first trimester and to those who smoked throughout the pregnancy), maternal diabetes (no diabetes, GDM in current pregnancy, type 1 diabetes by childbirth or type 2 or other diabetes by childbirth) and hypertensive disorders (normotension, gestational hypertension, pre-eclampsia or pre-existing/chronic hypertension), maternal weight (kg), and height (m) were extracted from Medical Birth Records (MBR). Body mass index (BMI) was calculated from the mothers' weight in kilograms divided by the square of height in meters. Metabolic risk factor was calculated as diabetes (absent vs. present), body mass index (BMI; 25 or less vs. >25) and hypertensive disorder during pregnancy (none vs. any) are highly intercorrelated (data not shown). The risk score reflected whether the mother had no metabolic risk factors or any (0 vs. 1). In addition, we conducted multidimensional scaling (MDS) analysis with PLINK and derived the MDS components from the IBS matrixes of the genotypes.

2.3 Statistical analysis

The effects of the *MTHFR* genotype on folate levels were analysed by multiple linear regression with the folate level as the outcome variable. The sum scores of the ASQ domains with either at least mild DD or FTD resembled Poisson count data and therefore the models with developmental outcome were performed using multiple Poisson regression.

I analysed models without covariates (crude model) and models with appropriate covariates for each analysis (adjusted models). Adjusted models with developmental outcomes included typically the following covariates: mother's education during pregnancy, mother's metabolic disorders during pregnancy using metabolic score, mothers smoking during pregnancy in three categories, child's sex, child's age when the ASQ-3 questionnaire was filled and gestational age. Further models with the genotype included MDS components as covariates. The model for the study question regarding the association between maternal folate levels and the genotype had only mother-related covariates affecting the folate metabolism including the following: mother's age, mother's education, mother's smoking, and first three MDS components.

Regarding the interaction models in addition to the crude model and the basic adjusted model a third model was conducted. It included basic covariates and further the cross-product terms of all covariates with both folate levels and with genotypes as suggested by Keller (2014) thus it is referred as Keller model. The covariates in Keller model were the same than in the basic adjusted excluding the MDS components and mother's smoking during pregnancy. Those had to be discarded from these Keller adjusted interaction models as their inclusion made the whole interaction model unstable because their standard errors were enormous leading to unreliable cross-product interaction terms thus affecting the reliability of the other parameters. For the significant interactions further analysis were conducted for the stratified groups by genotype.

Model fit was adequate for all Poisson models as the deviance value/df varied between 0.7 and 1.5 throughout all models and the Pearson chi-square value/df was over 1 and under 2 in all models. All the statistical analyses were done using SPSS (version 25.0, IBM Corp., Armonk, NY, USA) and R (version 3.5.2; R Core Team, 2018).

3. Results

As shown in Table 3, maternal folate levels at any measurement time during pregnancy or the mean folate level throughout the pregnancy were not associated with the child's risk to display either at least mild DD or FTD (p-values > .11).

Table 3. *The effect of maternal folate levels at different measurement times during pregnancy on the number of developmental milestone domains child displays at least mild developmental delay (DD) or failing to meet typical development (FTD).*

	mild DD			FTD		
	RR	[95% CI]	p	RR	[95% CI]	p
Crude model						
Early folate[b]	1.05	[0.88, 1.25]	.58	0.95	[0.70, 1.30]	.75
Mid folate[c]	1.04	[0.87, 1.24]	.69	0.88	[0.63, 1.23]	.46
Late folate[d]	1.00	[0.84, 1.19]	.97	0.84	[0.60, 1.16]	.28
Mean folate[e]	1.04	[0.87, 1.24]	.67	0.89	[0.65, 1.23]	.49
Adjusted model[a]						
Early folate[b]	1.03	[0.86, 1.22]	.77	0.86	[0.64, 1.15]	.31
Mid folate[c]	1.06	[0.88, 1.28]	.54	0.85	[0.61, 1.19]	.35
Late folate[d]	1.01	[0.85, 1.20]	.92	0.77	[0.56, 1.06]	.11
Mean folate[e]	1.05	[0.88, 1.26]	.57	0.85	[0.62, 1.15]	.29

Abbreviations: RR, relative risk; CI, confidence interval for RR

[a]*adjusted for:* Mother's education, mother's metabolic disorders during pregnancy, mother's smoking during pregnancy, child's gender, child's age when the ASQ questionnaire was filled and gestational age.

[b] n = 185

[c] n = 190

[d] n = 187

[e] n = 192

As shown in Table 4 the rs1801133 genotype did not associate with serum folate levels in the early measurement time (p-value = 06). However, the CT/CT genotype in rs1801133 was associated with a lower level of serum folate in the mid and late measurement point and with a lower mean folate level over all measurement times (p-values < 0.006). All of these associations remained significant after adjusting for covariates (p-values < 0.03). The rs1801131 AC/CC genotype was associated with a higher level of folate at all measurement points and a higher mean level throughout pregnancy in the crude models (p-values < 0.03) as well as in the adjusted models (p-values < 0.04). The rs1999594 genotype was not associated with serum folate levels at any measurement point.

Table 4. *The effect of MTHFR genotype on folate levels at different measurement times during pregnancy.*

	Folate level measurement time															
	Early pregnancy				Mid pregnancy				Late pregnancy				Mean level through out pregnancy			
	β	[95% CI]	p	n	β	[95% CI]	p	n	β	[95% CI]	p	n	β	[95% CI]	p	n
Crude model																
rs1801133 CT/TT vs. CC[b]	-0.09	[-0.19, 0.005]	0.06	399	-0.14	[-0.24, -0.04]	**0.005**	408	-0.15	[-0.24, -0.05]	**0.003**	399	-0.13	[-0.23, -0.04]	**0.006**	415
rs1801131 AC/CC vs. AA[b]	0.12	[0.03, 0.22]	**0.01**	399	0.13	[0.04, 0.23]	**0.01**	408	0.11	[0.01, 0.20]	**0.03**	399	0.13	[0.03, 0.22]	**0.01**	415
rs1999594 GA/GG vs. AA[b]	-0.04	[-0.14, 0.06]	0.44	399	-0.05	[-0.15, 0.04]	0.27	408	-0.08	[-0.17, 0.02]	0.13	399	-0.05	[-0.15, 0.04]	0.28	415
Adjusted model[a]																
rs1801133 CT/TT vs. CC[b]	-0.07	[-0.17, 0.03]	0.17	392	-0.11	[-0.20, -0.01]	**0.03**	401	-0.12	[-0.21, -0.02]	**0.01**	395	-0.1	[-0.12, -0.01]	**0.03**	407
rs1801131 AC/CC vs. AA[b]	0.12	[0.02, 0.21]	**0.02**	392	0.12	[0.03, 0.22]	**0.01**	401	0.1	[0.01, 0.19]	**0.04**	395	0.11	[0.02, 0.21]	**0.02**	407
rs1999594 GA/GG vs. AA[b]	-0.02	[-0.12, 0.08]	0.66	392	-0.03	[-0.13, 0.06]	0.5	401	-0.07	[-0.16, 0.03]	0.16	395	-0.04	[-0.13, 0.06]	0.45	407

Abbreviations: β, standardized beta; CI, confidence interval for standardized beta; p, p-value; n, sample size

[a]*adjusted for:* Mother's age, maternal education, mother's metabolic disorders during pregnancy, first three MDS components and mother's smoking during pregnancy

[b]reference category with an RR value of 1

Table 5. *The effect of maternal MTHFR on the number of domains child displays at least mild developmental delay (DD) or failing to meet typical development (FTD).*

	mild DD			FTD		
	RR	[95% CI]	p	RR	[95% CI]	p
Crude model						
rs1801133 CT/TT vs. CC[b]	1.03	[0.81, 1.31]	.82	1.43	[0.97, 2.11]	.07
rs1801131 AC/CC vs. AA[b]	0.78	[0.62, 0.99]	**.04**	0.61	[0.41, 0.91]	**.01**
rs1999594 GA/GG vs. AA[b]	1.18	[0.92, 1.51]	.19	1.25	[0.83, 1.87]	.28
Adjusted model[a]						
rs1801133 CT/TT vs. CC[b]	0.96	[0.75, 1.22]	.71	1.28	[0.86, 1.90]	.23
rs1801131 AC/CC vs. AA[b]	0.77	[0.60, 0.98]	**.03**	0.63	[0.42, 0.94]	**.02**
rs1999594 GA/GG vs. AA[b]	1.12	[0.88, 1.44]	.36	1.17	[0.78, 1.76]	.45

Abbreviations: RR, relative risk; CI, confidence interval for RR; p, p-value
[a]*adjusted for:* Mother's education, mother's metabolic disorders during pregnancy, mother's smoking during pregnancy, child's gender, child's age when the ASQ questionnaire was filled, three MDS components and gestational age.
[b]reference category with an RR value of 1
n = 456

Table 5 presents the effects of genomic variation in *MTHFR* on the two DD outcomes in the offspring. The maternal rs1801131 AC/CC genotype was associated with a lower risk of both at least mild DD or FTD compared to the AA genotype in the crude model ($p < .04$) and after adjusting for covariates ($p < .03$). Maternal rs1801133 and rs1999594 genotypes were not associated with either developmental outcome (p-values > .07).

Table 6 displays the interactions between maternal folate levels and the maternal *MTHFR* genotype on the number of domains the child displays at least mild DD or FTD. In the crude model the maternal rs1801131 genotype moderated the effect of maternal early pregnancy folate levels on both the child's risk of displaying at least mild DD (p for interaction = .002) and FTD (p for interaction = .01) more widely. After adjusting the basic covariates, the interaction remained significant for the child's mild DD outcome (p for interaction = .009) yet was only borderline significant for the child's FTD outcome (p for interaction = .056). When further adjusting for Keller cross-product terms the interaction was yet again significant for both outcomes (p for interactions < .02).

In the model further adjusted for the Keller cross-product terms, the maternal rs1801131 genotype also moderated the association between the maternal mean pregnancy folate level and the child's risk of displaying at least mild DD (p value for interaction = .047) and FTD (p =.03). The association was closing significance in the crude model for both outcomes (p for

interactions > .07) yet clearly non-significant in the basic adjusted model for both outcomes (p for interactions > .14).

Table 6. *P-values for the interaction between folate levels in different measurement times and maternal MTHFR on the number of domains child displays at least mild developmental delay (DD) or failing to meet typical development (FTD)*

	At least mild DD			FTD		
	Crude model	Adjusted model[a]	Keller models[b]	Crude model	Adjusted model[a]	Keller models[b]
Early pregnancy[d]						
rs1801133 CT/TT vs CC[c]	.19	.16	.07	.64	.82	.23
rs1801131 AC/CC vs. AA[c]	**.002**	**.009**	**.003**	**.01**	.06	**.02**
rs1999594 GA/GG vs. AA[c]	.64	.48	.28	.88	.99	.69
Mid pregnancy[e]						
rs1801133 CT/TT vs CC[c]	.15	.18	.07	.90	.78	.41
rs1801131 AC/CC vs. AA[c]	.39	.73	.25	.13	.23	.07
rs1999594 GA/GG vs. AA[c]	.26	.13	.22	.75	.76	.68
Late pregnancy[f]						
rs1801133 CT/TT vs CC[c]	.46	.44	.21	.30	.16	.89
rs1801131 AC/CC vs. AA[c]	.55	.66	.24	.34	.38	.07
rs1999594 GA/GG vs. AA[c]	.54	.31	.99	.77	.44	.90
Mean folate level[g]						
rs1801133 CT/TT vs CC[c]	.20	.21	.06	.92	.64	.44
rs1801131 AC/CC vs. AA[c]	.08	.17	**.05**	.07	.14	**.03**
rs1999594 GA/GG vs. AA[c]	.33	.16	.10	.99	.89	.83

Abbreviations: p, p-value

[a] *adjusted for:* Mother's education, mother's metabolic disorders during pregnancy, mother's smoking during pregnancy, child's gender, standardized child's age when the ASQ-3 questionnaire was filled, standardized gestational age and three MDS components.

[b] *adjusted for:* Mother's education, mother's metabolic disorders during pregnancy, child's gender, standardized child's age when the ASQ-3 questionnaire was filled, standardized gestational age and Keller cross-product terms of all covariates with both folate levels and with genotypes. These models do not include any MDS components or mother's smoking as they made the models unstable.

[c] reference category with an RR value of 1

[d] n = 178

[e] n = 182

[f] n = 179

[g] n = 184

Figure 1 illustrates that, in the models further adjusted for Keller cross-product terms, when maternal early pregnancy folate levels were low mothers having any C alleles had a lower risk of at least mild DD and FTD in the offspring compared to mothers carrying the AA genotype. Lower than 2SD folate levels from the sample mean were considered as low folate levels in the current study. Additional post-hoc comparisons further indicated that the difference between genotype groups was significant (data not shown). However, as seen from the Figure 1 no difference in the risk of at least mild DD or FTD in the offspring was

observed between mothers having any C alleles or carrying the AA genotype when maternal early pregnancy folate levels were average or high. Folate levels over 2SD higher than the mean were considered as high in the current study. Again, these results were supported by the additional post-hoc comparisons.

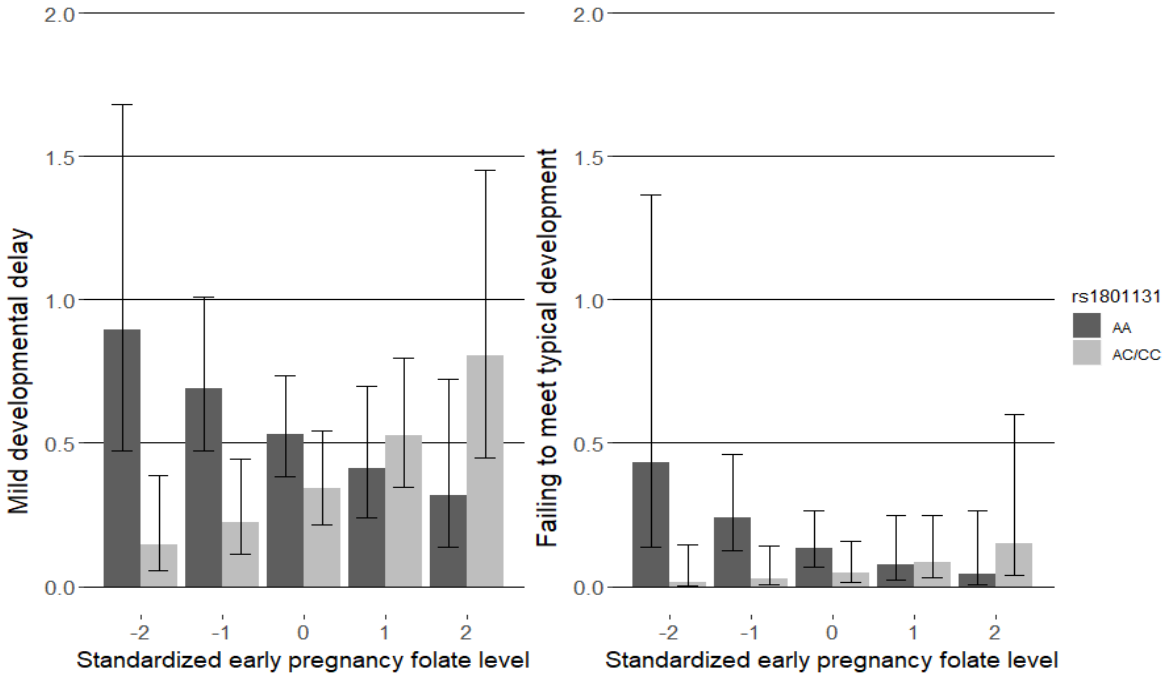

Figure 1. *Illustration of the simple main effects for maternal rs1801131 genotype groups of the association between maternal early pregnancy folate level and number of domains child display mild developmental delay (DD) (right) or failing to meet typical development (FTD) (left). For illustrative purposes simple main effects for the maternal rs1801131 are estimated at five different folate levels from the models adjusted for covariates from the Keller models.*

However, there was variation in the results of different interaction models which is seen when additional post-hoc comparisons were conducted for the other models containing significant interactions between maternal early pregnancy folate levels and maternal rs1801131, meaning the crude models with the child's risk of displaying at least mild DD and FTD as the outcomes and an adjusted model with the child's risk of displaying mild DD as the outcome. Those additional post-hoc comparisons yield similar results with low and average maternal folate levels than the Keller models meaning that with low folate levels mothers with any C alleles had a lower risk of DD in the offspring compared to mothers carrying the AA genotype and with average folate levels there was no difference between maternal rs1801131 genotype groups. However, with high maternal folate levels, mothers with any C alleles had a higher risk of DD in the offspring compared to mothers with the AA

genotype (data not shown), which is not in line with models adjusted further for Keller cross-product terms in which no difference was observed with high maternal folate levels.

Table 7. *The effect of maternal early pregnancy folate levels or maternal mean folate level throughout pregnancy on risk of child's mild developmental delay (DD) or failing to meet typical development (FTD) in number of developmental milestone domains separately on the genotype groups of maternal rs1801131.*

	rs1801131 genotype group							
	AA				AC/CC			
	RR	CI	p	n	RR	CI	p	n
Early pregnancy folate								
Mild DD	0.75	[0.55, 1.03]	.08	87	1.32	[0.99, 1.77]	.06	91
FTD	0.63	[0.36, 1.09]	.10	87	1.07	[0.61, 1.87]	.81	91
Mean folate								
Mild DD	0.91	[0.67, 1.23]	.52	89	1.26	[1.26, 1.66]	.11	95
FTD	0.66	[0.38, 1.17]	.16	89	1.13	[0.64, 1.97]	.67	95

Abbreviations: RR, relative risk; CI, confidence interval p, p-value; n, sample size
adjusted for: Mother's education, mother's metabolic disorders during pregnancy, mother's smoking during pregnancy, child's gender, child's age when the ASQ questionnaire was filled and first three MDS components.

From Figure 1 it is observed that the risk of mild DD and FTD in the offspring seems to decrease as the maternal early pregnancy folate levels increase with mothers having an AA genotype. This is also seen as a non-significant trend in the Table 7 containing separate analyses conducted in different maternal rs1801131 genotype groups. Figure 1 and Table 7 also indicates that mothers having any C alleles seem to have an increased risk of at least mild DD in the offspring when maternal early pregnancy folate levels increase, yet with mothers carrying any C allele the child's risk of FTD seems to be unaffected by the maternal early pregnancy folate levels. This is also seen in Table 7 as a closing significance trend of an association between maternal early pregnancy folate levels and the child's risk of mild DD but no association between maternal early pregnancy folate levels and the child's risk of FTD. Thus, the effect of high maternal early pregnancy folate level on child's risk of especially mild DD with mothers carrying any C allele is left somewhat uncertain in the light of these results.

As seen in the Figure 2 the interaction between maternal mean folate levels throughout pregnancy and maternal rs1801131 on the risk of at least mild DD or FTD in the offspring is similar than the observed interaction between maternal early pregnancy folate levels and maternal rs1801131 illustrated in the Figure 1 and explained in more detail earlier. However, Table 7 does not offer support to the association as all the effects are not even closing

significance (p > .11). This might be due to the differences in the adjusted covariates in the Keller model from which the illustrations in the Figure 2 are drawn and the separate analysis which only contains the covariates from the adjusted model and not the Keller cross-product terms as the analysis is done separately on the genotype groups. Thus, the results in separate analyses in Table 7 reflect the adjusted model interaction in the Table 6 which is non-significant unlike the Keller model. However, the similar findings between the interaction models adjusted further for Keller cross-product terms with both folate measures, the maternal early pregnancy folate and maternal mean folate level throughout pregnancy, offers additional support to the observed interaction.

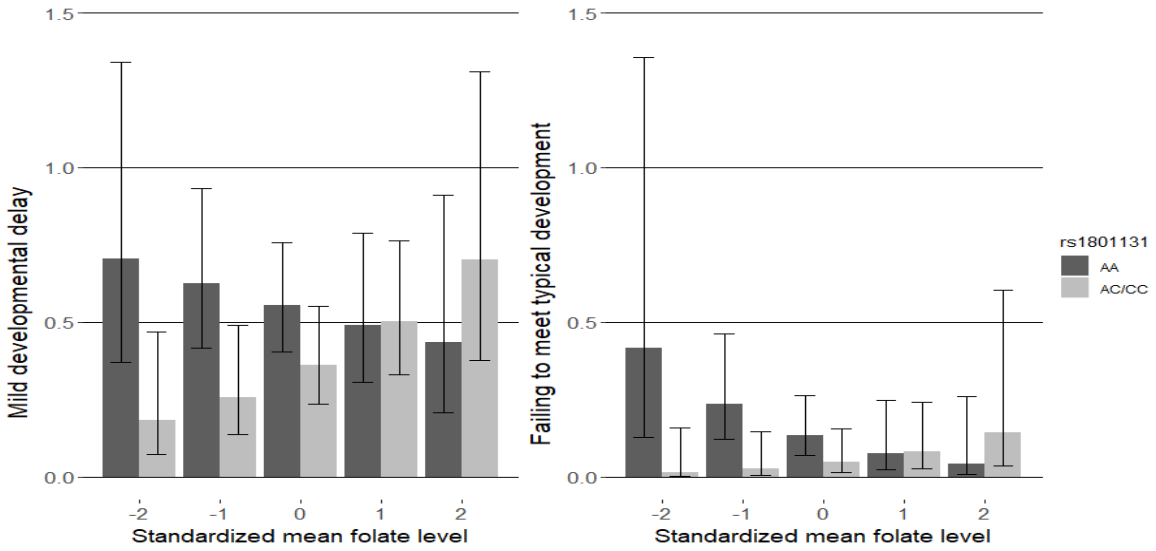

Figure 2. *Illustration of the simple main effects for maternal rs1801131 genotype groups of the association between maternal mean folate level throughout pregnancy and number of domains child display mild developmental delay (DD) (right) or failing to meet typical development (FTD) (left). For illustrative purposes simple main effects for the maternal rs1801131 are estimated at five different folate levels from the models adjusted for covariates from the Keller models.*

Interactions between maternal mid or late folate measurement times and maternal rs1801131 were not significant on either child's developmental outcome (p-values for interaction > .05). None of the interactions were significant between genomic variation in maternal rs1801133 or rs1999594 and any folate measurement time on either developmental outcome variable.

4. Discussion

The aim of this study was to assess whether prenatal folate level and maternal genetic variants associated with folate metabolism affect the child's risk of DD and whether the association between maternal prenatal folate and child's DD is moderated by the maternal *MTHFR* genotype.

The current study did not detect a direct association between maternal folate levels at any time during pregnancy and the risk of displaying at least mild DD or FTD in the offspring. This is partly in agreement with previous studies as measures of folate levels during late gestational weeks have not been associated with the child's subsequent neurocognitive development (Tamura, 2005; Wu et al., 2012). On the other hand, contradictory to our findings, relatively early pregnancy folate level measures have been previously associated with later neurocognitive development (Schlotz et al., 2010; Steenwegde et al., 2012), yet not consistently (Steenweg-de GraaffJ et al., 2014). DD is a severe developmental disorder thus it is possible that enough folate during pregnancy may enable at least typical development. The majority of the mothers in the current sample had enough folate, thus rooftop effect may prevent any difference to be seen between mothers who have low or high folate levels as continuous variable was used for this study. Group comparisons would have probably been statistically underpowered, due to the low frequency of mothers with low folate deficit (Table 2), and thus were not conducted in this study.

Considering the second study question, the rs1801133 CT/TT genotype was associated with lower serum folate levels compared to the CC genotype. This is in line with previous GWA (Deng et al., 2018; Grarup et al., 2013) and enzyme activity (Frosst et al., 1995; van der Put et al., 1998) studies. The association was evident on mid and late pregnancy folate levels and on the mean folate level across measurement times. No association was found between the early pregnancy folate level and rs1801133. This might be explained by the evident increased folate levels during early pregnancy probably due to national recommendation to increase folate intake during first trimester of pregnancy (THL, 2018). Increased folate intake has been found to almost fully recover the damaged enzyme function caused by the T allele in rs1801133 (Marini et al., 2008). Therefore, current findings can be interpreted to support the previous findings indicating that rs1801133 affects folate metabolism especially when folate levels are low and increased folate levels can compensate the lowered MTHFR activity

(Marini et al., 2008). Furthermore, the sensitivity of rs1801133 to folate intake might explain why it has not been constantly associated with folate (Hazra 2009; Tanaka, 2009).

Continuing, the AC/CC genotype in rs1801131 was associated with higher serum folate levels compared to the AA genotype which is in line with a previous meta-analysis (Yu et al., 2018). Yu et al. (2018) proposed that the association might be mediated trough impaired folic acid conversion due to lowered MTHFR enzyme activity associated with an additional C allele. Folic acid can be absorbed in the liver as such, thus with high intake some of it can be left circulating unmetabolized and may be seen as higher folate levels in serum (Pietrzik, Bailey, & Shane, 2010; Scaglione & Panzavolta, 2014). As folic acid must be converted to a biologically active form by enzyme dihydrofolate reductase (DHFR) before use slow conversion may lead to increased amount of unmetabolized folic acid in the serum (Pietrzik et al., 2010; Scaglione & Panzavolta, 2014) which may be seen as higher folate levels. However, MTHFR enzyme is not directly involved in the conversion of the folic acid and as Yu et al. (2018) did not propose any other mechanism on how the rs1801131 genotype would affect the conversion the association proposed by Yu et al. (2018) is highly hypothetical. Also, a question remains how genomic variants with comparable effects on enzyme activity could associate with opposing folate levels as the T allele in rs1801133 is also linked with decreases in MTHFR activity but decreased folate levels as discussed earlier.

Another explanation might be the one suggested by Parle-McDermott et al. (2006) that accelerated rate of conversion from 5,10- methyleneTHF to 5-methylTHF might explain the association. However, they associated rs1801131 only with red cell folate (RCF) not with plasma folate and also poined out that the mechanism is still uncertain. A highly hypothetical mechanism could be that as rs1801131 is part of a regulatory area of *MTHFR* (Weisberg et al., 1998) the C allele may actually increase the amount of the MTHFR enzyme available by increasing the expression of *MTHFR* gene. This might over-compensate the decreased activity previously observed (van der Put et al., 1998; Weisberg et al., 2001; Weisberg et al., 1998) and thus lead to increased folate levels. Observation of a trend linking the C allele with an increased expression of the *MTHFR* gene compared to the A allele in many tissues supports the need for future research on this subject (GTExPortal, 2019).

Another interesting discussion is the possible additive genetic effect of rs1801133 and rs1801131 on folate levels as the two SNPs are in high LDE. In each chromosome the harmful allele in the other SNP nearly always links with the beneficial allele in the other SNP

thus leading a person unlikely to be homozygous for both alleles connected to lowered enzyme activity (van der Put et al., 1998; Weisberg et al., 2001). Therefore, when studying separately these SNPs the observed beneficial effect of the C allele in rs1801131 might actually reflect the beneficial effect of the C allele in rs1801133. Highlighting the importance of studying the additive effect of these two SNPs comes from the finding that the rs1801131 C allele do not seem have an independent effect on folate levels, but when taking into account the allele combination in rs1801133 the C allele in rs1801131 further lowers the folate levels (van der Put et al., 1998). It means that heterozygous for both rs1801133 and rs1801131 seem to have lower folate levels compared to those heterozygous for only rs1801133 and homozygous for rs1801131 (van der Put et al., 1998).

Finally, for the second study question, no association between rs1999594 and serum folate levels were observed in this study. Even though rs199594 was the strongest SNP associated with folate levels in a relatively small GWAS it did not reach genome-wide significance and thus it might have been a coincidence GWAS (Tanaka et al., 2009). Previous findings have associated rs1999594 with homocysteine levels, which was not measured in the current study (Hazara, 2009; Paré et al., 2009). Even though, homocysteine and folate levels are found to be inversely associated (Selhub, Jacques, Wilson, Rush, & Rosenberg, 1993) the current study does not suggest association between rs199594 and folate levels.

As for the third study question, the current study found no associations between maternal rs1801133 or maternal rs1999594 and the child's risk of at least mild DD or FTD. The lack of association between maternal rs1801133 and offspring neurodevelopmental outcomes is in contrast with the earlier study associating rs1801133 to child's mental development (Pilsner et al., 2010). In the current study the association between maternal rs1801133 and the risk of FTD in the offspring was closing significance ($p = .07$). The number of children displaying FTD is rather small, thus a lack of variation might have weakened the statistical power of the analysis and explaining why only a borderline significant effect was detected. Yet, as the association between maternal rs1801133 and child's FTD weakened further after adjusting for covariates this discussion is highly hypothetical.

In addition, the current study observed an association between maternal rs1801131 and the risk of at least mild DD and FTD in the offspring. The additional maternal C allele was found to be beneficial as it was associated with a decreased risk of mild DD or FTD in the offspring. To my knowledge, this is the first study to detect this association between maternal

rs1801131 and child's development as the two previous studies considering maternal rs1801131 and child's mental development did not detect any associations (del Río Garcia et al., 2009; Pilsner et al., 2010). Ethnic differences between previous samples and the current sample might explain why no associations have been detected previously. Previous studies (del Río Garcia et al., 2009; Pilsner et al., 2010) were conducted on Mexican populations, which have lower frequency of the C allele compared to European populations including Finns (Guéant-Rodriguez et al., 2006) and thus might not have enough genetic variation in the rs1801131 to detect any association. However, our finding is not completely unexpected as prior evidence does exist, considering the connection between the child's rs1801131 genotype and their neurocognitive development (Pu et al., 2013). The association is probably mediated by the positive effect of any maternal C allele on prenatal folate levels which somehow benefits the child's development during pregnancy.

As for the fourth and final study question, no interaction was observed between maternal rs1801133 or rs1999594 and any measurement time folate level on the child's risk of DD. This is partly in line with the previous studies as Pilsner et al (2009) did not detect significant interaction and del Río Garcia et al. (2009) only detected borderline significant interaction between folate intake and rs1801133 on child's mental development index. However, the current findings are not in line with Gatica-Domínguez et al (2019) study as they detected an inverse association between increased folate levels and child's mental developmental index in CC-carrier mothers but not in any other maternal rs1801133 genotype group (p-value for interaction < .10). However, their interpretations should be considered with caution as they used a 10% risk level instead the typically used 5%. Differences in the samples might partly explain the lack of association in the current study compared to theirs. Gatica-Domínguez et al. (2019) had a high number of mothers having folate levels over the recommendation of WHO, compared to no mother having folate levels that high in the current sample (WHO limit according to Gatica-Domínguez et al. (2019) converted to nmol/l: 20ng/ml * 2.266 = 45.32 nmol/l, see Table 1 for comparison). This might be partly due to a food fortification program present Mexico but not in Finland. Ethnical differences in the allele frequencies might also affect as they had a high number of mothers with the TT genotype (32%) as expected in Mexican population (Gatica-Domínguez et al., 2019) compared to the European sample used in the current study (see Table 1 for comparison).

To my knowledge, the current study is the first to find a moderating effect of the maternal rs1801131 on the association between the maternal early pregnancy folate and the risk of DD in the offspring. Having any maternal C alleles seems to be a protecting factor for both mild DD and FTD in the offspring when maternal early pregnancy folate levels are low. The weak statistical power due to the small sample size and especially small number of children displaying FTD coupled with a complex model might explain why the association was not evident in all models. A similar protective effect of any maternal C allele was also found with low maternal mean folate levels throughout pregnancy on the risk of the child displaying at least mild DD and FTD. The observation of a similar trend offers additional support on the beneficial effect of any maternal C allele when folate levels are low even though the moderation of the maternal rs1801131 on the association between maternal mean folate level throughout pregnancy and the child's DD is more uncertain.

Investigating the interaction further suggests that maternal early pregnancy folate levels seem to not affect the risk of FTD in the offspring with mothers carrying any C alleles. With those mothers the risk of FTD in the offspring seems to stay at the same level throughout different levels of maternal early pregnancy folate levels. On the other hand, increasing folate levels seems to benefit children whose mothers carry the AA genotype even to the extent that the risk of FTD in the offspring whose mothers carry the AA genotype might be even lower than with those whose mothers carry any C allele, yet that observation is somewhat uncertain as it is not evident throughout models.

There is a similar effect of a decreased risk of at least mild DD in the offspring when maternal early pregnancy folate levels increase in children whose mothers carry the AA genotype. However, mothers who carry any C allele seem to also have an increased risk of at least mild DD in the offspring when folate levels increase. This might suggest some disadvantage of any maternal C allele combined with high maternal early pregnancy folate level. Increased amounts of unmetabolized folic acid due to an impairment of folic acid conversion caused by any C allele, as suggested by Yu et al. (2018), might explain the current observation. The "methyl trap hypothesis" suggests that high folate levels due to unmetabolized folic acid could mask the deficiency of vitamin B12, thus leading to adverse outcomes (Pietrzik et al., 2010). However, the mechanism through which rs1801131 might affect folic acid conversion is not known and further the current evidence suggests that the intake of the recommended amount of folic acid is safe and should not mask the deficiency of

vitamin B12 (Crider, Bailey, & Berry, 2011). Adverse outcomes in the offspring have previously been detected only when maternal prenatal folic acid intake is approximately 5 to 10 times over the recommended amount of 400µg per day (Valera-Gran et al., 2014). As this study did not record folate intake the hypothesis cannot be completely discarded, yet as Finland does not have food fortification and folic acid is obtained only from supplements it is highly unlikely that any mother had too high intake by accident. Further all the mothers in the current sample had folate levels under the upper limit thus too much of folic acid is a highly unlikely explanation. It remains uncertain how the maternal rs1801131 C allele together with high maternal prenatal folate levels might be connected to a higher risk of at least mild DD in the offspring and replications are highly needed to strengthen the association before placing further hypotheses.

There are some limitations in the current study. Although the sample size is similar to the previous study with a similar aim (Gatica-Domínguez et al., 2019), the sample size is still rather small, thus the analysis may still be underpowered to detect all associations. The sub samples used in this study are only a proportion of the original PREDO sample, thus selection bias might have some effect. For example, those mothers with early pregnancy obesity, maternal diabetes, or hypertensive disorders during pregnancy were over-represented in the sample. Although a variety of well-known risk factors for DD were statistically controlled, residual confounding may be an issue as for example we were not able to control for maternal alcohol consumption during pregnancy.

As there were only a few mothers having folate deficiency, making a group comparison statistically underpowered and continuous variable was selected for this study. Despite the major limitations caused by the small sample size, group comparisons with the current sample might provide some further information of the possible association. However, that kind of further analyses are beyond the scope of this study.

The ASQ-3 is a good tool for screening DD, yet it also places some limitations to the current study. As developmental delay is a rare condition, only few children displayed FTD. In future studies samples with a higher prevalence of severe developmental delay are needed to investigate the association more closely. The mild DD variable has a higher prevalence than FTD thus offering more variation between the children in the sample, yet it is a quite troublesome outcome. The at least mild DD includes children whose score is between -1SD to -2SD from the sample mean which is still considered to be within the broad variation of

normal developmental although displaying some concern of possible delay. Furthermore, the mild DD outcome does not distinct the severity of the DD. A child who have a score of -1SD from the mean in one domain and a child who have a score of -2SD from the mean in one domain both have a sum score of one in the mild DD variable even though the first one is only possibly delayed and the later one is displaying severe delay. Regardless of the problems in the mild DD variable the observation of similar effect as with the FTD outcome does offer additional support for the current findings. Also, future studies with more variety in the neurodevelopmental outcomes and measures capable to distinguish also among typically developed would provide further evidence of the association between prenatal folate and the neurodevelopment during childhood.

Objective measurement of folate level and multiple measurement times during pregnancy are major strengths in the current study. In future it would also be interesting to separate different folate isoforms from the serum to enable closer inspection of the possible effect of unmetabolized folic acid on child development. Also, recording the intake of folic acid and combining that data to the information of the serum folate ratio and further to genetic information would enable closer inspection of the possible moderating effects of genetic variation in maternal *MTHFR* on the child's development and further whether genetic variation in *MTHFR* affects the conversion of folic acid compared to naturally occurring folate. Future studies are also needed to establish the mechanism of how rs1801131 affects the folate metabolism. Further studies could also inspect whether maternal folate levels or maternal *MTHRF* genotype associate with global methylation in cord blood as folate is a key part of methylations.

To my knowledge, the current study is the second study to examine the interaction between prenatal maternal folate levels and maternal rs1801133 polymorphisms on child's development and the first to include the two other *MTHFR* SNPs rs1801131 and rs1999594. Further, this study is the first to study the interaction between maternal *MTHFR* and maternal prenatal folate levels on child's neurocognitive development in the European population. The current study replicated the previous findings associating the rs1801133 T allele with lower folate levels and the rs1801131 C allele to higher folate levels. To my knowledge, this is the first study to detect an association between maternal rs1801131 to child's risk of DD and even further an interaction between maternal rs1801131 and maternal prenatal folate levels on child's DD. These findings suggest that the maternal C allele in rs1801131 seems to be

beneficial to a child's development when folate levels are low compared to mothers carrying the AA genotype. However, mothers carrying the AA genotype seem to benefit from increased maternal folate levels even to the extent that the risk of DD in the offspring might be lower with high maternal folate levels compared to mothers carrying any C allele. Some evidence suggests that high maternal folate levels combined with mothers having any C alleles might also increase the child's risk for at least mild DD, yet that finding is highly uncertain and demands replications. Thus, despite some limitations, the current study offers interesting evidence of the moderating effect of the maternal genetic variation associated with folate metabolism on prenatal folate levels affecting the risk for DD in the offspring.

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